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Manganese transport through human erythrocyte membranes. An EPR study

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Abstract

Manganese uptake by human erythrocytes was investigated in the concentration range 0.5–20 mM in the suspending solution, by using the EPR technique. S shaped dependencies of manganese influx on manganese doping solution concentration for both fresh and vanadate treated erythrocytes were found, with maximum influx values of $4.1 \pm 1.9 \times 10^{-10}$ mol/m$^2 \times$ s and $2.1 \pm 0.3 \times 10^{-9}$ mol/m$^2 \times$ s, respectively. At low manganese concentrations (< 2 mM) the manganese permeability coefficient increases with increasing the doping concentration, the ions cooperate for achieving a transport event. For high manganese concentration (> 5 mM) the permeability coefficient decreases with increasing the doping concentration, the ions competing for the limited amount of transport system. A similar increase in manganese uptake as in vanadate treated erythrocytes was measured for 'in vitro' aged erythrocytes. These results might suggest that human erythrocytes possess an active transport mechanism by which, they oppose to manganese influx. This hypothesis is also supported by the 10–15 min time lag between the moment of doping and the start of the manganese influx into the fresh erythrocytes. The manganese uptake inhibition by nifedipine, a calcium channel blocker, for the case of vanadate treated erythrocytes, suggests that, at least partially, manganese uptake by the cells occurs via the 'calcium channels'. © 1997 Elsevier Science B.V.

Keywords: Manganese; Erythrocyte; EPR

1. Introduction

The manganese permeability of the human red blood cell membranes was measured in the early 60's by using a radioisotopic method [1]. It was found that manganese enters the cells via a passive diffusion mechanism, with a permeability coefficient of 2.87×10^{-11} m/s. Due to the apparent chemical instability of their radiomanganese stock solutions, the authors of this study limited the divalent cation concentration to up to 0.5 mM and excluded molecular oxygen from the system.

The interest for the manganese divalent ion increased when it was suggested that it could be conveniently used as a calcium probe, as it is now well established that in many species and cellular systems it is transported through the cell membranes via the calcium transport pathways. Manganese is taken up

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by mitochondria over the calcium uniporter [2] and penetrates into rat ileal cells [3], rat peritoneal mast cells [4], bovine chromaffin cells [5], insect muscle membranes [6], mouse ovarian oocyte [7], myoepithelial cells of a spongi [8], sarcolemma of the rat heart [9], sarcoplasmic reticulum of rabbit skeletal muscle [10], human platelets [11], rat hepatocytes [12] via the calcium channels. Rat astrocytes transport manganese via a specific transport system, which is not inhibited by other divalent cations (Co^{2+}, Zn^{2+}, Pb^{2+}) and it is reduced only in the presence of 2–3 order of magnitude higher Ca^{2+} concentrations [13]. On the other hand it was demonstrated that passive calcium uptake by the human erythrocytes is mediated to a large extent by a 'Ca^{2+} channel' being inhibited by calcium channel antagonists and divalent cations such as Zn^{2+}, Cu^{2+}, Co^{2+} and Cd^{2+}[14].

Due to its paramagnetic properties, Mn^{2+} was extensively used as a paramagnetic probe in NMR experiments. Conlon and Outhred [15] have proposed a NMR technique, now frequently used, for measuring the water exchange times through the erythrocyte membranes by using up to 40 mM manganese as a magnetic label of the external cell environment. A prerequisite of the method is that the added manganese ions do not penetrate through the cell membranes. However, as it was demonstrated in our laboratory [16,17], this condition holds true only for the first 10–15 min of the experiment, in whole blood or in samples doped with low manganese concentrations. In washed erythrocytes and/or at high manganese doping concentrations the NMR data can be strongly influenced by the paramagnetic ions permeation through the cell membranes [18].

On the other hand, it was demonstrated that the water exchange times through the human erythrocyte membranes, measured by using the NMR manganese doping technique are modified in cells from patients with various pathologies [19,20]. These results could be interpreted also as changes in the manganese permeability of the cells in certain pathologies.

For all these reasons it was felt that a systematic investigation of the manganese transport into human erythrocytes in a broad range of manganese concentrations and in the presence of known inhibitors was necessary.

In this paper we used an electron paramagnetic resonance (EPR) method for measuring the manganese uptake by the human red blood cells. The EPR technique is very sensitive to changes in the paramagnetic cation binding and/or changes in the oxidation state [21] and it was also applied for measuring manganese uptake by mitochondria[22]. Our method is based on the fact that manganese doped hemolysates do not exhibit detectable EPR signals. As a consequence, the EPR signal of a manganese doped suspension of red blood cells is related to the outer cell paramagnetic ions. A decay in the EPR signal amplitude is therefore explained by the cation penetration into the cells and subsequent binding. We measured the manganese uptake by fresh, 'in vitro' aged and vanadate treated erythrocytes, as well as the effects of temperature and of Ca^{2+} entry blockers on manganese uptake for a large range of manganese doping concentrations (0.5–20 mM). We performed measurements at these high concentrations mainly for two reasons: (i) these are the usual concentrations used in NMR experiments for measuring the water permeability of red blood cells and (ii) we presupposed that manganese enters the cells via the calcium transport pathways, which saturate at concentrations above 5 mM.

A preliminary abstract of a part of this work has been published (Copăescu, L.I., Dragu, C. and Lucaciu, C.M. (1996) Progr. Biophys. Mol. Biol. 65 Suppl. I, 100.).

2. Materials and methods

2.1. Materials

N-2-Hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) was from Sigma Chemicals Co., manganese (II) chloride, ethylenediamine tetraacetic acid (EDTA) and sodium vanadate were from Merck and nifedipine from Terapia, Ltd. All solutions were made in doubly distilled water. All other chemicals were of analytical grade.

2.2. Blood preparation

Human blood obtained by venipuncture from apparently healthy donors was collected on either EDTA or heparin and used within 2 h. Whole blood was centrifuged (Janetzki T-24) for 10 min at $1000 \times g$.

After centrifugation plasma and buffy coat were carefully removed by aspiration. Subsequently the red blood cell pellet was washed three times in an isotonic solution buffered at pH 7.4 (145 mM NaCl; 5 mM KCl, 5 mM HEPES). The final centrifugation lasted 10 min, the supernatant and the top 0.5 ml red blood cell being removed by aspiration.

The samples for EPR were prepared prior to the measurement by thoroughly mixing an aliquot of washed erythrocytes with an appropriate volume of isotonic doping solution containing variable amounts of manganese chloride, buffered at pH 7.4.

In parallel, 0.5 ml of the doped suspension were centrifuged in hematocrit tubes at $2000 \times g$ for 30 min. The ratio between the cell pellet volume and the whole suspension volume, corrected for the trapped water, was used as the probe hematocrit in the subsequent calculations.

Varecka and Carafoli [23] have shown that preincubation of human red blood cells with 0.5 mM vanadate, leads to the inhibition of the red blood cell active transport system after permeation of the membrane through the anion exchange system. They used this method to study Ca^{2+} passive influx into the red blood cell.

Engelmann and Duhm [14] have shown that vanadate reaches its maximum effect at or above 2 mM. We incubated the erythrocytes at 37°C for 30 min in HEPES buffered saline supplemented with 2 mM vanadate, i.e. with arrested active transport systems. After this treatment the cells were washed again and we measured the manganese influx for various manganese concentrations.

Nifedipine was dissolved in absolute ethanol and this solution was added to the doping solution. In preliminary experiments it was checked that 2% ethanol in the suspending medium does not change significantly manganese transport through the red blood cell membranes. The experiments with nifedipine were carried out in the dark.

2.3. EPR measurements

The doped suspension of cells was aspirated in a glass capillary tube and mounted in the resonance chamber of an X-band EPR spectrometer (ART-6, IFIN Bucharest). The spectrometer resonance chamber was thermostated at 37 ± 1°C by using air flow. The spectrometer settings were: microwave frequency 9.01 GHz, modulation frequency 100 kHz, modulation field 2 Gs.

The EPR spectra were continuously recorded. The data were analyzed by simply measuring the amplitudes of the first derivative six lines Mn^{2+} spectra, furnished by the spectrometer. We previously checked that, up to 100 mM $MnCl_2$ in HEPES buffered saline, the amplitude of the divalent manganese EPR spectrum increases linearly with the manganese chloride concentration (coefficient of determination better than 0.99).

2.4. Manganese binding to cell hemolysate

In preliminary experiments for the titration of manganese binding to intracellular compounds, washed erythrocytes were frozen in liquid nitrogen and then thawed at 37°C. This cycle was repeated three times. After 30 min of centrifugation at $2500 \times g$ the hemolysate was diluted 5 times in double distilled water. No membrane fragments were seen under these conditions in the supernatants by using dark field microscopy. Equal volumes of hemolysate solutions were mixed with manganese (II) chloride stock solutions and HEPES buffered at pH 6.9. The EPR technique was used to measure the divalent manganese binding to intracellular compounds. Measurements were made in the same glass capillary tube, mounted always in the same position in the spectrometer resonance chamber. The divalent manganese EPR signals for doped hemolysates were compared with those of manganese chloride standards measured in the same conditions. The hemolysate concentration was expressed in units of hemoglobin concentration, the major cytoplasm protein, which was measured by using the cianmethemoglobin method.

Due to their restricted rotational molecular motion, paramagnetic ions bound to macromolecules present a strong increase, beyond the detection limit, in the width of the EPR signals [21]. Thus for manganese chloride doped hemolysates, only the free manganese ions will contribute to the observable EPR signal. From the difference between the total manganese concentration, known from the preparation, and the EPR measured free divalent ion concentration, we obtained the bound manganese concentration.

We analyzed the data by using the Scatchard Klotz equation:

$$1/\mu = K_D/[n\mathrm{Mn}_f] + 1/n \quad (1)$$

were μ is the average number of metal ions bound per molecule of protein, n is the number of metal binding sites (assumed to be equivalent and independent), K_D is the dissociation constant and $[\mathrm{Mn}_f]$ is the free manganese ion concentration. From the plot $1/\mu$ versus $1/[\mathrm{Mn}_f]$, which was linear, we obtained a dissociation constant of $K_D = 3.36 \pm 0.43$ mM and a number of binding sites $n = 16.2 \pm 1.3$. These results emphasize the large binding capacity of the red blood cell inner components.

Riddell and Zhou [24] have not observed any change with time in the width of the intracellular $^{35}Cl^-$ NMR line in human erythrocytes, in the presence of extracellular Mn^{2+}. As the NMR line broadening of $^{35}Cl^-$ is very sensitive to the addition of small concentrations of Mn^{2+} this result is a very strong evidence for extremely tight binding of intracellular Mn^{2+} supporting our findings.

At this moment it is not clear the ligand to which manganese binds when added to hemolysates, but is presumable that several molecules can contribute to this effect. It was demonstrated [1] that 60% of the manganese taken up by the red blood cells or added to hemolysates are not ultrafilterable and no saturation of the manganese binding sites was observed for concentrations ranging from 10^{-7} M to 10^{-3} M. Gupta and Benovic [25] have found, by using the EPR technique, that 2,3-bisphosphoglycerate binds manganese ions with a dissociation constant $K_D =$ 0.75 mM, having two binding sites. Other red blood cell molecules which bind tightly manganese are ATP, ADP ($K_D \sim 0.1$ mM) [25]. We expressed the hemolysate concentration in units of hemoglobin which has a molecular mass of several orders of magnitude higher than other potential manganese ligands. Therefore our value for the dissociation constant is only a mean, which is seen for all these molecule types, but having a practical use in estimating the fraction of free metal ions when added to cell hemolysates.

With these values for K_D and n we have calculated that, at the highest manganese concentration used in this study (i.e. ~ 20 mM), at equilibrium, for a 50% hematocrit suspension, the free manganese concentration inside the cell will contribute with less than 5% to the overall EPR signal. Therefore, for all practical reasons, the inner cell manganese can be considered bound to intracellular compounds. The detected EPR signals of manganese doped suspensions of red blood cells were attributed to the outer cell divalent manganese ions.

Weed and Rothstein [1] have reported a fast manganese association with the red blood cells, which they attributed to the cation binding to the cell membranes. They found that about 5% from the doping, which was less than 0.5 mM, was associated with the cells in red cell suspensions or with red cell isolated membranes. Getz et al. [26] have measured the EPR signals of 5 mM manganese doped suspensions of erythrocytes and they did not observed changes in the Mn^{2+} EPR signal amplitude or shape. They concluded that manganese ions do not bind to red blood cell membranes. In the conditions used in our study (relatively high manganese concentrations, about 50% hematocrit) the binding of a small amount of manganese to red blood cell membranes is masked by the decrease in the manganese concentration caused by the dilution due to the water trapped between the cells.

At high manganese concentrations and for long incubation times hemolysis occurs and this phenomenon can influence the concentration of the free manganese ions and subsequently the divalent manganese EPR signal amplitude, leading to artefactual permeability data. We reduced the incubation times (usually we used 30 min) to reduce the influence of hemolysis (which was less than 1%) on the permeability data.

Weed and Rothstein [1] have measured a day to day change in the manganese uptake by the red blood cells, which they attributed to the oxidation of the radiomanganese stock solutions. However, polarographic determinations revealed only small changes in the Mn^{2+} concentrations of their stock solutions, which could not account for the significant day to day changes in the manganese uptake by the cells. High hemolysis rates and possible divalent manganese oxidation and/or precipitation when incubating cells for long times (> 3 h) at high (> 5 mM) manganese concentrations were also reported [1].

As the EPR method is very sensitive to changes in the state of the paramagnetic ions, Mn^{2+} oxidation,

precipitation or binding can be very easily monitored by using this technique. We did not measured significant changes in the EPR divalent manganese signal amplitude of our $MnCl_2$ stock solution during several days. No such types of difficulties were reported by an other group of investigators who measured the Mn^{2+} EPR signal of manganese doped suspension of erythrocytes [26].

Using two different techniques, EPR and atomic absorption spectroscopy (AAS) we have demonstrated that the decrease in the manganese concentration of the manganese doped erythrocyte supernatants parallels the decrease observed in the Mn^{2+} EPR signal, at least for the first 30 min of incubation (Lucaciu C.M., Bindea C. and Morariu V.V., unpublished data). For longer incubation periods hemolysis can affect the EPR data, leading to artefactual higher permeability coefficients. However, as in AAS both Mn^{2+} and Mn^{3+} are detected and the EPR technique detects only Mn^{2+}, we concluded that no significant oxidation of the divalent manganese occurs in the conditions used in our experiments.

2.5. Data analysis

The data were interpreted in terms of a two compartment model, intra and extracellular, considering that only the diffusion through the membrane is the rate limiting factor. Having in mind that inside the cells, the manganese ions are bound and nondiffusable, we obtained, after a straightforward integration, the time dependence of the number of extracellular manganese ions:

$$N = N_o \times \exp(-k \times t) \quad (2)$$

or

$$N = N_o \times \exp[-P \times H \times S \times t/V \times (1-H) \quad (3)$$

where N is the initial number of manganese ions, k is the kinetic coefficient, P is the permeability coefficient, H the suspension hematocrit and S and V are the normal erythrocyte surface area and volume, respectively.

Since the inner cell manganese ions are bound and nondiffusable, we have considered that the volume in which the ions diffuse is infinity. Under these circumstances the equation shows that for very long incubation times the extracellular manganese ion concentration tends to zero, i.e. the divalent cations accumulate into the cells.

The manganese ions influx into the cells was calculated as the product between the permeability coefficient and the manganese doping solution concentration.

3. Results

Following the EPR signal of the divalent manganese ion added to a suspension of human erythrocytes we observed a gradual decrease in the six lines signal amplitudes. As it was discussed above, this decrease is related to manganese uptake by the red blood cells and subsequent binding to inner cell components. No significant changes in the shape of the Mn^{2+} six lines spectra were detected. However, for very long incubation times, after a drastically decrease in the signal amplitude, the modulation of the six lines Mn^{2+} spectrum with a large line ($\sim$ 300 Gauss) was observed. It seems that, in these cases, there is a superposition of the isolated extracellular Mn^{2+} aqueous ions signal with a signal arising from a small fraction of strongly dipole-dipole interacting Mn^{2+} ions. This fact may indicate that, inside the erythrocytes, there are small pools with free manganese ions, having a high local concentration.

Some typical recordings are given in Fig. 1. In certain cases the decrease in the Mn^{2+} occurs only

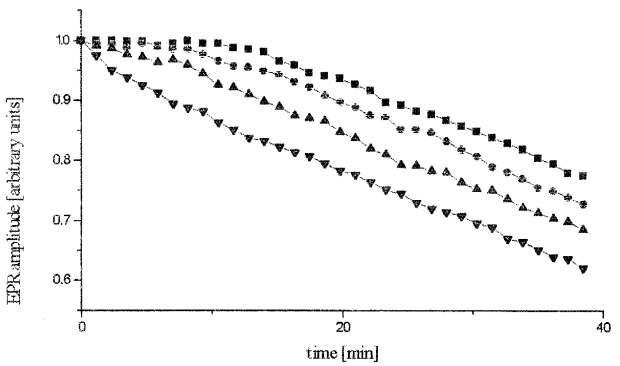

Fig. 1. Time dependence of the Mn^{2+} EPR signal amplitude added to red blood cell suspensions from the same washed red blood cell pellet doped successively at 1 hour time intervals in the order: ■, ●, ▲, ▼. Manganese doping concentration was 5 mM for each sample.

after 10–15 min of incubation. This observation suggests that the cells possess a mechanism by which, in certain conditions, they oppose to the manganese influx, for a short time at the beginning of the influx experiment. This phenomenon did not occurred in every sample. We could not made a clear cut correlation for this type of behavior with the preparation procedure steps, or with the doping manganese concentration.

For samples, from the same washed red blood cell pellet, doped successively at 1 h time intervals, we obtained influx curves having similar slopes, but with significant differences in the first part of the influx experiment (Fig. 1). The cell pellet was kept at room temperature until doping each sample. While for the first sample the decrease in the Mn^{2+} EPR signal occurs only after about 10–15 min of incubation, for the subsequent dopings this time interval is reduced. For the last sample no such a behavior can be detected. For these reasons, care was take to measure the manganese influx within a short time interval after the blood was collected.

We obtained large differences between the permeability coefficients for blood samples from different donors (up to 2–3 fold). For samples from the same blood, kept at 4°C, and washed successively, we obtained for the permeability data a mean coefficient of variation of about 10%. Within the same coefficient of variation (12.3%, $n = 4$) were also the permeability coefficients measured for the same blood sample in the conditions mentioned above, at different hematocrit values (in the range 20–60%). This fact allows us to consider that the hematocrit correction in Eq. (2) was legitimate.

We found that manganese influx dependence on manganese doping solution concentration is S shaped (Fig. 2). This type of dependence is characteristic for a cooperative transport mechanism [27]. The permeability coefficient dependence on manganese doping solution concentration presents a maximum at about 2 mM. For lower manganese concentration the permeability coefficient increases with the increase of the manganese concentration. In this case, two or more manganese ions cooperate to achieve transport. At higher manganese concentration the permeability coefficient decreases as the doping solution concentration increases, competition for the limited amount of transport system was found.

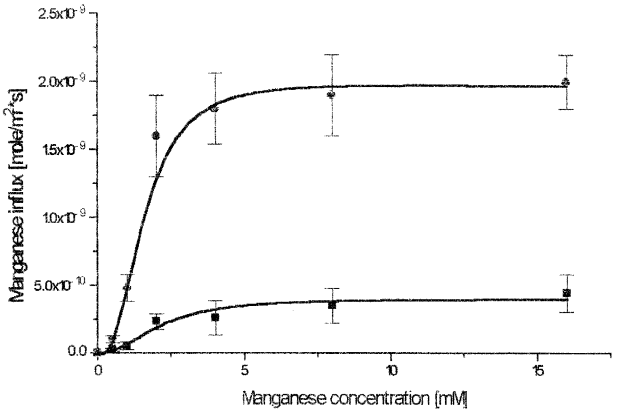

Fig. 2. Manganese influx dependence on $MnCl_2$ doping solution concentration for (■) fresh erythrocytes and (●) vanadate treated erythrocytes. Each point represents the mean of more than 10 samples. Bars indicate standard deviations. The dependence is S shaped for both cases, denoting cooperativity. The solid lines are the best fittings of the experimental data with Eq. (3) from which we obtained maximum influx values of $2.1 \pm 0.3 \times 10^{-9}$ mol/m^2 $\times$s and $4.1 \pm 1.9 \times 10^{-10}$ mol/m^2 $\times$s for vanadate treated and fresh erythrocytes, respectively.

The concentration dependence of the influx was fitted with the following equation [27]:

$$J = C \times (J_1 \times C + J_2)/(C^2 + K_1 \times C + K_2) \qquad (4)$$

where C is the manganese doping concentration J_1, J_2, K_1 and K_2 are the parameters which characterize the transport system. We obtained for the maximum influx at limitingly high values of C, $J_1 = 4.1 \pm 1.9 \times 10^{-10}$mol/m^2 $\times$ s and for the permeability coefficient at limitingly low values of C (i.e. 0.5 mM) $P_{min} = 3.1 \pm 1.1 \times 10^{-11}$ m/s.

'In vitro' aging of the erythrocytes was obtained by 24 h incubating the cells in their plasma, at different temperatures. We followed the manganese influx after washing and doping in similar conditions as for fresh erythrocytes. The influx curves were exponential and well fitted by Eq. (3). We obtained a gradual increase of the manganese influx, depending on incubation temperature. For cells incubated at 37°C the maximum influx was 3.4 ± 0.4 times ($n = 10$) higher than the influx measured for the same sample in 1 h after the blood was collected. This increase may be explained either by ATP depletion or to the various biochemical changes which occur in aged cells [28].

The time dependence of Mn^{2+} EPR signal amplitude for manganese doped suspensions of vanadate treated red blood cells are exponential (Fig. 3) and the manganese influx dependence on manganese doping solution concentration is S shaped (Fig. 2). Fitting the experimental data with Eq. (3) we obtain a maximum influx $J_{max} = 2.1 \pm 0.3\ 10^{-9}$ mol/m^{2}s, which is about 5 fold higher than in controls. This strong increase in the manganese uptake by vanadate treated erythrocytes supports the idea that the red blood cells possess an ATP dependent mechanism, by which they can oppose to the manganese influx.

We measured the temperature dependence of manganese uptake by fresh human erythrocytes in erythrocyte suspensions doped with high manganese concentration, near saturation (18 mM) at different temperatures. The Arrhenius plot is linear in the temperature range investigated (4–37°C), giving an activation energy of 117 ± 15 KJ/mol.

Nifedipine is known as a calcium channel blocker. For concentrations up to 0.3 mM it was found that nifedipine decreases manganese uptake by vanadate treated erythrocyte (Fig. 3). The effect depends on nifedipine and manganese concentrations. In the presence of 0.3 mM nifedipine the manganese uptake is

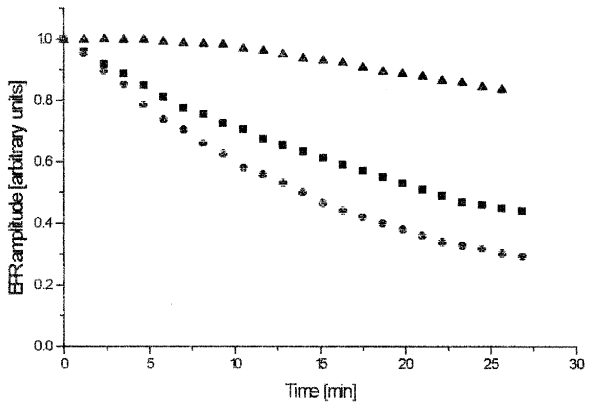

Fig. 3. Time dependence of the Mn^{2+} EPR signal added to a suspension of red blood cells. (▲) Fresh erythrocytes, (●) cells pretreated by incubation 30 min at 37°C in isotonic saline supplemented with 2 mM sodium vanadate, (■) vanadate treated erythrocytes with 0.3 mM nifedipine in the doping solution. For all three cases suspensions hematocrit was about 0.45 and the erythrocytes were doped with 5 mM $MnCl_2$ and incubated at 37°C in the resonance chamber of the spectrometer.

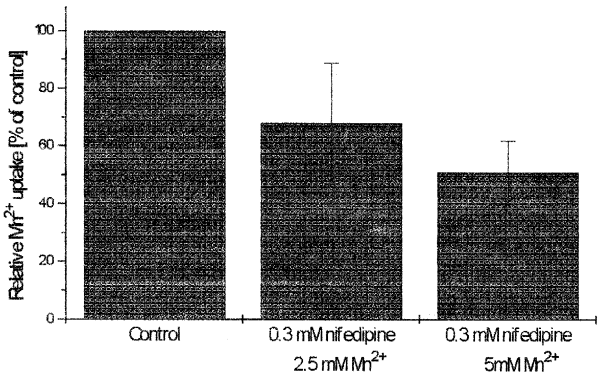

Fig. 4. Manganese influx inhibition by nifedipine. Nifedipine (0.3 mM), dissolved in ethanol, was added in the doping medium. The ratios between the manganese influx in the presence of nifedipine and the manganese influx for the controls for two manganese doping concentrations are presented. Bars indicate standard deviations, $n = 4$ for both cases.

$51 \pm 11\%$ and $66.3 \pm 21\%$ from the controls for manganese dopings of 5 mM and 2.5 mM, respectively (Fig. 4).

4. Discussion

Strong evidences have been brought in previous works from our laboratory [17] for the fact that the divalent manganese ions, added to a suspension of human erythrocytes can penetrate through the cell membranes, affecting the inner cell proton relaxation times. The time decay of the water protons long relaxation time, measured in paramagnetically doped suspensions of cells, can be used to measure the permeation of the paramagnetic ions through the cell membranes. Stout et al. [29] have found, by using the NMR technique, that the divalent manganese ions influx in *Elodea* leaf cells is about 3×10^{-10} mol/m$^2 \times$ s, being independent on the external manganese concentration in the range 10–80 mM. However, in the case of the human erythrocytes, quantitative determinations are difficult to made by using this technique, mainly for two reasons: (i) the proton relaxation time depends nonlinearly on the divalent manganese concentration [30], (ii) the binding of the divalent paramagnetic cations to macromolecules leads to a significant enhancement of the proton relaxation [21,30]. Nevertheless, qualitative evaluations and comparative studies on the effect of drugs

and pathologies on manganese uptake by human erythrocytes have been performed by using the NMR technique[18,31].

A parallel NMR and atomic absorption spectroscopic (AAS) study from our laboratory [31] has revealed that manganese concentration into the cells increases almost linearly in time, but starting after 10–15 min from the beginning of the experiment. This behavior might be explained either by a cell resistance to manganese influx at the beginning of the experiment, or to a wrong evaluation of the very beginning of the experiment. An other possible explanation of these findings is the under evaluation of all inner cell concentrations, due to divalent ions binding to the cell membranes, as only the cytosolic manganese was measured. It was difficult to draw clear-cut conclusions about the first 10–15 min of influx by using these methods, due to their low time resolution.

In the EPR method presented in this paper we were able to monitor the manganese influx within a few minutes from the start of the doping procedure, followed by measurements at 1 min time interval. As the decay in the Mn^{2+} EPR signal amplitude occurs, for some samples, after 10–15 min of incubation, our EPR data clearly indicate that the cells oppose themselves for a while to the manganese influx.

The permeability coefficients obtained at 0.5 mM manganese concentration, i.e. $3.1 \pm 1.1 \times 10^{-11}$ m/s are in agreement with those obtained by Weed and Rothstein [1] for similar concentrations (2.87×10^{-11} m/s). However, extending the range of the manganese doping concentrations, we obtained that manganese permeability increases with concentration up to about 5 mM and then decreases. A similar behavior was noticed for vanadate treated erythrocytes, in this later case the influx values being about 5 times higher. This type of dependence implies some cooperative mechanism by which the ions at low concentrations collaborate for achieving a transport event [27]. At higher manganese concentrations (above 5 mM) the permeability coefficient decreases with increasing concentration. In other words the product between the permeability coefficient and the doping concentration tends to a constant. In terms of manganese influx this means saturation.

The value of the activation energy is two times higher than that measured by Weed and Rothstein [1] for low manganese concentrations (up to 0.5 mM). This difference in the activation energy, obtained in the two studies, might by explained either by a direct influence of the high cation concentration on the transporter or, as it was discussed above, to the cooperative mechanism by which two or more cations cooperate to achieve a transport event.

It is questionable whether manganese ions are transported inside the red blood cells via the calcium transport systems. It is known that calcium transport into the red blood cells have mainly two components: a saturable flux component of about 50 μmol/l cell $\times$ h (i.e. $\sim 10^{-11}$ mol/m^{2}s), which is reduced by ATP depletion and has at its turn two components, selectively inhibited by different Ca^{2+}-channel entry blockers [14]; a linear flux component, which corresponds to a permeability coefficient of less than 2×10^{-13} m/s. On the other hand human erythrocytes possess a strong ATP consuming Ca pump, which is able to extrude calcium outside the cells with a rate of 10–25 mmol/l cell $\times$ h (i.e.$2–5 \times 10^{-9}$ mol/m$^2 \times$ s) [32].

The manganese influx reported in this paper is about 2–3 orders of magnitude higher than Ca^{2+} influx in human erythrocytes. The idea that manganese is transported into the red cells via the same transporter as calcium is conceivable with this discrepancy if we suppose that the transporter has a 2–3 order of magnitude higher affinity for manganese than for calcium. It was reported that 1mM Ca (a concentration which is also the half saturation constant for calcium influx) reduces manganese influx into the red blood cells to 50%, for very low manganese concentrations. (10^{-5} M) [1]. In other words a 2 order of magnitude higher calcium concentration can significantly inhibit manganese influx into red blood cells. On the other hand, at least partially, calcium influx in human erythrocytes occurs via a ‘calcium channel’, being reduced in the presence of calcium channels antagonists. It is known that manganese is transported in many excitable cells via the calcium channels or other calcium transporters[2–12]. Our results on manganese influx in the presence of nifedipine support the hypothesis that manganese can penetrate the red blood cell membranes through ‘calcium channels’.

The increase in the manganese influx for ‘in vitro’ aged erythrocytes, correlated with the same effect

observed in vanadate treated cells, led us to hypothesis that human erythrocytes possess an ATP dependent mechanism by which they can face the manganese ions influx. From this point of view, the manganese uptake measured without vanadate treatment in fresh erythrocytes is only the balance between passive manganese influx and active extrusion. In vanadate treated cells the active extrusion being blocked, what we measure is only the passive diffusion influx.

One mechanism by which the cells oppose to manganese influx might be Ca^{2+}-Mg^{2+} ATP. The difference between the manganese influx in vanadate treated erythrocytes and fresh cells matches the maximum pumping rate of the calcium pump. It is known that manganese can activate the red blood cell ATPases either from the cytosol or from the cell membranes [33,34] and it modulates the Sr^{2+} transport through the calcium pump [35]. At the same time it was demonstrated that manganese is transported through the calcium pump of the sarcoplasmic reticulum [36]. For other mammalian species it was demonstrated that manganese is transported into erythrocyte through the Na-Ca exchanger [37], which lacks in human erythrocytes.

We believe that our results support the idea that manganese can be transported through the human erythrocyte calcium pump at least at the concentrations used in our study. However, further experiments are needed to prove this hypothesis as well as to extrapolate these findings to physiological concentrations.

References

[1] R.I. Weed, A. Rothstein, J. Gen. Physiol. 44 (1960) 301–313.
[2] T.E. Gunter, R.N. Rosier, D.A. Tucker, K.K. Gunter, Ann. NY Acad. Sci. 307 (1978) 246–247.
[3] T. Nasu, H. Arakaki, H. Shibata, Gen. Pharmacol. 26 (1995) 1255–1260.
[4] D. Falcone, C. Fewtrell, J. Cell. Physiol. 164 (1995) 205–213.
[5] R.I. Fonteriz, J. Garcia-Sancho, L. Gandia, M.G. Lopez, A.G. Garcia, Am. J. Physiol. 263 (1992) C818–824.
[6] J. Fukuda, K. Kawa, Science 196 (1977) 309–311.
[7] S. Yoshida, J. Physiol. 339 (1983) 631–642.
[8] M. Anderson, J. Gen. Physiol. 81 (1983) 805–827.
[9] D.R. Hinter, R.A. Haworth, H.A. Berkoff, J. Mol. Cell. Cardiol. 13 (1981) 823–832.
[10] T. Sekiguchi, S. Kawahara, H. Shimizu, J. Biochem. 102 (1987) 307–312.
[11] M.T. Alonso, A. Sanchez, J. Garcia-Sancho, Biochem. Biophys. Res. Commun. 162 (1989) 24–29.
[12] G.E. Kass, J. Llopis, S.C. Chow, S.K. Duddy, S. Orrenius, J. Biol. Chem. 265 (1990) 17486–17492.
[13] M. Aschner, M. Gannon, H.K. Kimelberg, J. Neurochem. 58 (1992) 730–735.
[14] B. Engelmann, J. Duhm, Biochim. Biophys. Acta 981 (1989) 36–42.
[15] T. Conlon, R. Outhred, Biochim. Biophys. Acta 288 (1972) 354–361.
[16] V.V. Morariu, M.S. Ionescu, M. Frangopol, R. Grosescu, M. Lupu, P.T. Frangopol, Biochim. Biophys. Acta 815 (1985) 189–195.
[17] V.V. Morariu, M.S. Ionescu, M. Frangopol, R. Grosescu, M. Lupu, P.T. Frangopol, Biochim. Biophys. Acta 860 (1986) 155–158.
[18] V.V. Morariu, M.S. Ionescu, M. Frangopol, R. Grosescu, M. Lupu, P.T. Frangopol, Biochim. Biophys. Acta 900 (1987) 73–78.
[19] V.V. Morariu, G. Benga, Biochim. Biophys. Acta 288 (1977) 354–361.
[20] V.V. Morariu, L. Petrov, Cancer Biochem. Biophys. 45 (1986) 199–202.
[21] R.A. Dwek, NMR in Biochemistry, Clarendon Press, Oxford, 1973.
[22] R.E. Gunter, J.S. Puskin, P.R. Russell, Biophys. J. 15 (1975) 319–333.
[23] L. Varecka, E. Carafoli, J. Biol. Chem. 257 (1982) 7414–7421.
[24] F.G. Riddell, Z. Zhou, Magn. Res. Chem. 33 (1995) 66–69.
[25] R.K. Gupta, J.L. Benovic, Biochem. Biophys. Res. Commun. 84 (1978) 130–137.
[26] D. Getz, J.F. Gibson, R.N. Sheppard, K.J. Miklem, C.A. Pasternak, J. Membrane Biol. 50 (1979) 311–329.
[27] W.D. Stein, in: Methods in Enzymology, Vol. 171, Academic Press, 1989, pp. 23–62.
[28] A. Brovelli, C. Seppi, C. Balbuini, Int. J. Biochem. 16 (1983) 1115–1120.
[29] D.G. Stout, R.M. Cotts, P.L. Steponkus, Can. J. Bot. 55 (1977) 1623–1631.
[30] P. Marzola, S. Cannistraro, Physiol. Chem. Phys. Med. NMR 18 (1986) 263–273.
[31] V.V. Morariu, T. Simplaceanu, C. Bindea, A. Chifu, P.T. Frangopol, Rev. Roum. Biochim. 25 (1988) 239–244.
[32] L.V. Lew, in: Intracellular Calcium Regulation, Alan R. Liss, Inc., 1990, pp. 1–17.
[33] H. Pfleger, H.U. Wolf, Biochem. J. 147 (1975) 359–361.
[34] M.H. Mostafa, D.J. Hanahan, Biochim. Biophys. Acta 802 (1984) 490–500.
[35] E.J. Olson, J. Membrane Biol. 48 (1979) 265–284.
[36] A. Gomes da Costa, V.M. Madeira, Arch. Biochem. Biophys. 249 (1986) 199–206.
[37] M.D. Frame, M.A. Milanick, Am. J. Physiol. 261 (1991) C467–475.